Repeated evolution of asexuality involves convergent gene expression changes

Darren J. Parker[1,2,*], Jens Bast[1], Kirsten Jalvingh[1], Zoé Dumas[1], Marc Robinson-Rechavi[1,2], and Tanja Schwander[1]

1. Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland

2. Swiss Institute of Bioinformatics, Lausanne, Switzerland

* Corresponding author: DarrenJames.Parker@unil.ch

Abstract

Asexual reproduction has evolved repeatedly from sexual ancestors across a wide range of taxa. While the costs and benefits associated with the proliferation of asexual lineages has received considerable attention, the molecular underpinnings of the changes required for asexual reproduction remain relatively unexplored. In particular, it is completely unknown whether the repeated evolution of asexual phenotypes involves similar molecular changes, as previous studies have focused on changes occurring in single lineages. Here we investigate the extent of convergent gene expression changes and patterns of molecular evolution across five independent transitions to asexuality in stick insects. We compared gene expression of asexual females to those of females from close sexual relatives in whole-bodies and two tissues: the reproductive tract and legs. We identified a striking amount of convergent gene expression change, ranging from 5 to 8% of genes examined, greatly exceeding that expected by chance. Convergent changes were also tissue-specific, with most convergent genes changing in only one tissue type, as expected from selection for functional changes. Conversely, lineage-specific changes were not tissue-specific, as expected from drift. Functional enrichment tests found that genes showing convergent changes in the reproductive tract were associated with meiotic spindle formation and centrosome organization. These genes are particularly interesting as they can influence the production of unreduced eggs, a key barrier to asexual reproduction. Changes in legs and whole-bodies were likely involved in female sexual trait decay, with enrichment in terms such as sperm-storage and pigmentation. By identifying changes occurring across multiple independent transitions to asexuality, our results provide a rare insight into the molecular basis of asexual phenotypes and suggest that the evolutionary path to asexuality is highly constrained, requiring repeated changes to the same key genes.

Keywords: convergent evolution, parthenogenesis, *Timema*

Introduction

Sexual reproduction is extremely costly. Sex is less efficient than asexuality for transmitting genes to future generations (Maynard Smith 1971) and in order to outcross, an individual has to find a partner, forgo foraging, and risk contracting sexually transmitted diseases and predation while mating (Bell 1982; Lehtonen et al. 2012). Yet, the overwhelming number of sexual, as compared to asexual, animal and plant species (Avise 2008; van der Kooi et al. 2017) indicates that sexual reproduction is highly advantageous. Identifying potential advantages conferred by sex has motivated decades of research and a rich body of work on the evolution and maintenance of sexual and asexual reproduction has been produced (reviewed in Bell 1982; Lewis 1987; West et al. 1999; Otto 2009; Neiman et al. 2017)). By contrast, little is known about the molecular underpinnings of transitions between reproductive systems (Neiman et al. 2014). Yet these molecular underpinnings have the potential to provide insights into the processes involved in the evolution of asexuality, and to help understand how sex is maintained. For example, sex is more easily maintained if asexuality evolves gradually in a sexual population than if it emerges suddenly via major effect mutations (Templeton 1982; Burt 2000; Schwander et al. 2010).

Some insight into the genetic basis of asexuality has been gained from studies of individual asexual lineages (Innes and Hebert 1988; Lynch et al. 2008; Eads et al. 2012; Jaquiéry et al. 2014), but a broad comparative framework for exploring common principles of the molecular basis of asexuality is lacking. For example, a major unresolved question is whether independent transitions to asexuality involve similar or different molecular changes. To address these shortcomings, we explored the molecular underpinnings of asexuality in stick insects of the genus *Timema*, a genus of wingless, herbivorous insects native to the West coast of North America and the mountains of the Desert Southwest. This group is uniquely suited for comparative studies of asexuality, as asexuality has evolved at least seven times independently (Schwander et al. 2011; Fig. 1), allowing us to study convergence across replicate transitions from sexual to asexual reproduction. Furthermore, close sexual relatives are at hand for each asexual lineage for comparison. All asexual *Timema* species reproduce via obligate parthenogenesis (Schwander and Crespi 2009), meaning that they evolved the ability to produce unreduced eggs which develop without fertilization by sperm. Additional phenotypic changes evolved convergently as adaptations to asexuality, including a reduced sperm storage organ, and reduced sexual pheromone production (Schwander, Crespi, et al. 2013). Thus,

73 asexual *Timema* females are less attractive to sexual males (Schwander, Crespi, et al. 2013),
74 which use both airborne and contact signals to identify suitable mates (Nosil et al. 2007;
75 Arbuthnott and Crespi 2009; Schwander, Arbuthnott, et al. 2013), and even when copulations
76 between sexual males and asexual females are forced under laboratory conditions, eggs are
77 not fertilized (Schwander, Crespi, et al. 2013).
78
79 To capture molecular changes associated with the evolution of asexuality we performed whole-
80 body and tissue-specific transcriptome sequencing (RNA-seq) on females from five sexual and
81 five asexual *Timema* species (Fig. 1). We chose two different tissues, the reproductive tract and
82 legs, to identify the molecular mechanisms underlying the production of asexual offspring
83 (reproductive tract), and adaptations to a celibate life (e.g. reduction of various different sexual
84 traits in the reproductive tract and legs). Note that the reproductive tract and leg samples
85 actually represent a collection of tissues, but we refer to them as tissues throughout for brevity.
86 Whole-body samples were included as they allow us to identify important changes that may be
87 missing in the tissue-specific transcriptomes. Using this approach, we identified convergent
88 expression changes which were likely driven by selection. We also observed changes specific
89 to each sexual-asexual species-pair which typically showed concerted changes across tissues,
90 consistent with being a product of drift (Blekhman et al. 2008; Liang et al. 2018). Finally, to
91 complement our expression analyses, we examined patterns of molecular evolution in genes
92 showing convergent expression changes following a transition to asexuality.
93
94
95
96

Results

Transcriptomes and orthology

Reference transcriptome assemblies for each species were generated previously (Bast et al. 2018). Bast et al. (2018) also identified 3010 one-to-one orthologs, which were used as our transcriptome reference. For each tissue, orthologs with low expression (counts per million less than 0.5 in two or more libraries per species) were filtered prior to expression analyses. Thus, the final number of orthologs kept for analyses of whole-body, reproductive tract, and leg samples was 2984, 2753, and 2740, respectively.

Convergent gene expression changes

We identified convergent gene expression changes between sexual and asexual species by modelling gene expression as a function of species-pair (see Fig. 1), reproductive mode (sexual or asexual), and their interaction in edgeR (Robinson et al. 2010). In such a model, convergence is indicated by an overall effect of reproductive mode (FDR < 0.05), but no interaction (FDR > 0.05) (Supplemental Table 1). Approximately four times as many genes changed convergently in the reproductive tract (7%; 203/2754) and legs (8%; 206/2737) as compared to the whole-body (2%; 57/2985), perhaps reflecting the relative difficulty in identifying expression changes in complex tissue assemblies such as whole-bodies (Johnson et al. 2013). The amount of convergence we observe is considerable and approximately double what we would expect by chance, for all tissues (whole-body: $p = 0.0128$, reproductive tract: $p < 0.0001$, legs: $p < 0.0001$, Supplemental Fig. 1). The amount of change between sexual and asexual females was relatively small for convergent genes, with a mean fold change of approximately 1.4 (absolute $\log_2$ expression change for whole-body = 0.55, reproductive tract = 0.68, and legs = 0.46) (Fig. 2).

As expected for selection-driven gene expression changes (Blekhman et al. 2008; Liang et al. 2018), convergent changes between sexual and asexual species were highly tissue-specific. Only 22 of the convergent genes in the reproductive tract (203) and legs (206) overlapped between the two tissues, a value not significantly greater than expected by chance (Table 1). There was also little overlap between convergent genes in the two tissues and whole-bodies (Table 1, Supplemental Fig. 2). This supports the interpretation that the convergent changes in

131 expression are driven by selection rather than by drift, as drift is more likely cause similar
132 changes across multiple tissues. This interpretation of selection also predicts that convergent
133 genes will be involved in divergent functions in each tissue which, as we show below, is indeed
134 the case.
135
136 ***Functional processes of convergently expressed genes***
137
138 To detect convergence at the process level, we performed gene set enrichment analyses
139 (GSEA). Briefly, we scored Gene Ontology (GO) terms according to the rank of convergent
140 expression change of genes annotated to the terms; GO terms were then called significant if
141 they had a better average rank than expected by chance (see Methods). More than 100 GO
142 terms are enriched in each tissue studied (FDR < 0.05), providing strong support for
143 convergence of biological processes between asexual species (Supplementary Tables 2-4).
144 This signal is not dependent on any threshold at the gene level, and thus provides information
145 on convergence at the process level due to small but consistent contributions from many genes.
146 Consistent with the gene expression results, enriched GO terms were generally tissue specific;
147 we found no significant overlap between GO-terms enriched in the legs and reproductive tract
148 (11 shared terms, FDR = 0.123), between the legs and whole-body (4 shared terms, FDR =
149 0.799), or between whole-body and reproductive tract samples (10 shared terms, FDR = 0.064).
150
151 To reduce the number of enriched GO terms to examine we semantically clustered enriched GO
152 terms using ReviGO (Supek et al. 2011) (Supplemental Tables 5-7). The annotations of
153 convergent changes in the reproductive tract reflect the convergent evolution of
154 parthenogenesis in asexual *Timema*, as they were linked to meiosis (meiotic spindle
155 organization, meiosis II, centrosome duplication, meiosis I cytokinesis, meiosis II cytokinesis),
156 and reproduction (growth of a germarium-derived egg chamber, sperm individualization, gamete
157 generation). However, convergent changes were also linked to neuron development
158 (neurogenesis, neuron development, neuron recognition), as well as several GO terms involved
159 in development and metabolic processes for which the link to asexuality is less clear. In legs we
160 identified GO terms involved in immune defence (response to fungus, regulation of production of
161 molecular mediator of immune response, regulation of antimicrobial peptide production,
162 regulation of humoral immune response), which may be because asexual females are no longer
163 susceptible to the costs associated with diseases transmitted from sexual interactions (Knell
164 and Webberley 2004). Convergent changes were also linked to sex determination (primary sex

165 determination; soma, primary response to X:A ratio), which may control changes in the
166 expression of sexual traits, and several metabolic processes. In whole-body samples we find
167 some reproduction associated terms (courtship behavior, male mating behavior, male courtship
168 behavior, sperm storage, regulation of ovulation) as in the reproductive tract, and behavioral,
169 and immune related terms (immune response-regulating cell surface receptor signaling
170 pathway) as in legs, but also some unique terms relating to the cuticle (ecdysone, pupal chitin-
171 based cuticle development).
172
173 ***Convergently expressed genes in whole-bodies show evidence for sexual trait decay***
174
175 Several of the enriched functional processes described above are suggestive of sexual trait
176 decay. Under this scenario we expect a reduction of purifying selection on genes underlying
177 sexually dimorphic traits in asexual species, indicated by an increased accumulation of non-
178 synonymous changes.
179
180 The power to detect differences in pN/pS or dN/dS between gene sets in asexuals is low, as
181 genes are inherited as a single linkage group. Nevertheless, we found that genes showing
182 convergent changes in expression in whole-bodies showed elevated pN/pS and dN/dS when
183 compared to the genomic background (permuted t-test p-value for: pN/pS < 0.0001, dN/dS =
184 0.0084, Fig. 3, Supplemental Figs. 3 and 4), consistent with the idea of sexual trait decay.
185 Sexual trait decay is further supported by the examination of functional annotations for such
186 genes which include one gene (OG-2854) that is produced primarily in male accessory glands in
187 *Drosophila*, and at least three other genes (OG-2197, OG-663, OG-1014) that are involved in
188 pigment synthesis pathways (pigmentation is sexually dimorphic in *Timema* see Fig. 1B). In
189 contrast, genes showing convergent changes in expression in the reproductive tract and legs
190 did not show elevated pN/pS or dN/dS (Fig. 3, Supplemental Figs. 3 and 4), suggesting that
191 convergent expression changes in these tissues do not coincide with reduced purifying selection
192 acting on their sequences.
193
194 We conducted several additional analyses to check the robustness of our results and
195 corroborate our interpretations. Firstly, we examined in detail the functions of candidate gene
196 sets for which there was very strong evidence for convergent changes, and secondly, we used
197 cross-species mapping to examine expression changes occurring across the whole
198 transcriptome, rather than only in the subset of genes we identified as single copy orthologs

199 between the 10 species. Both approaches support the results from our original analyses and are
200 described below.
201
202 ***Strongly convergent candidate genes and their function***
203
204 Although all the convergent genes we identified showed an overall shift in expression across the
205 five species-pairs, often expression change in one or two of the pairs was small (<1.2 fold
206 change). We defined top candidate genes as convergent genes for which the absolute $\log_2$ fold
207 change in expression was more than 0.25 (~1.2 fold change) for all species-pairs. Most of these
208 top genes showed convergent shifts in the reproductive tract (36 genes, relative to 4 and 15
209 genes for whole-body and legs, respectively) (Figure 4, Supplemental Table 1). The functions of
210 these candidate genes largely reflected the functional processes identified for the full set of
211 convergently expressed genes, and highlight a number of key genes potentially involved in
212 producing asexual phenotypes.
213
214 For the reproductive tract four genes are involved in meiotic spindle formation and centrosome
215 organization (OG-513, OG-1448, OG-1488, OG-314). In particular we find two genes (OG-1448,
216 OG-1488) belonging to a family of Elovl proteins that mediate elongation of very-long-chain fatty
217 acids, including an ortholog to *D. melanogaster* gene *bond*, which effects spindle formation and
218 has been shown to be important for meiotic, but not mitotic, cytokinesis (Szafer-Glusman et al.
219 2008). In particular, *D. melanogaster* males defective for *bond* commonly display two to four
220 nuclei in spermatids causing sterility. Female *bond* mutants are also infertile (Szafer-Glusman et
221 al. 2008), although the mechanism is unknown. The other two genes (OG-513, OG-314) have
222 roles in centrosome function, including an ortholog to *poc1* which is involved in centrosome
223 formation (Blachon et al. 2009). Six genes (OG-758, OG-2002, OG-1478, OG-1993, OG-2686,
224 OG-148) were annotated with reproduction associated terms which may be responsible for the
225 convergent reproductive changes we observe between asexual and sexual females.
226 Interestingly, one gene, OG-511, is an ortholog to glucose dehydrogenase which is important for
227 sperm storage in female *D. melanogaster (Bloch Qazi et al. 2003)*. Finally, we find that 11
228 genes (OG-1195, OG-1478, OG-1841, OG-2197, OG-2808, OG-366, OG-445, OG-511, OG-
229 705, OG-712, OG-758, OG-810) have annotations to the nervous system. The majority of these
230 appear to be sensory in nature, and in particular seven are annotated with the GO term
231 "sensory perception of pain". Changes in these genes may represent changes associated with
232 female receptivity and post-mating behaviour in asexual females, which are target of

233 substances in the male ejaculate (Heifetz and Wolfner 2004; Sakai et al. 2009; Heifetz et al.
234 2014).
235
236 For leg samples three genes (OG-1651, OG-2048 and OG-1081) are involved in immune
237 defence. In particular orthologs of both genes (*Trx-2* and *MP1*) are involved in the activation of
238 melanisation in response to fungal and bacterial infection in *D. melanogaster* (Tang et al. 2006;
239 Jin et al. 2008). Three genes are involved in cuticle development (OG-2221, OG-2738, and OG-
240 2995). Orthologs of two other genes (OG-1371 and OG-2031) are involved in male specific
241 behaviours (male courtship behavior and inter-male aggressive behavior) in *D. melanogaster*
242 (*CaMKII* and *Fkbp14*) (Mehren and Griffith 2006; Edwards et al. 2009). Since these genes are
243 also expressed in females, changes to their expression may have resulted from the release of
244 intralocus sexual conflict.
245
246 Whole-body samples had only four strong candidate genes, and all either have no annotation or
247 have only broad GO-terms annotated. One potentially interesting gene, OG-2188, has an
248 ortholog (*CG12237*) that has been associated with female sterility in *D. melanogaster* (Sopko et
249 al. 2014). Finally, the remaining candidate genes across all tissues were either unannotated (12
250 genes) or only have very broad GO-terms annotated (10 genes).
251
252 ***Cross-species mapping***
253
254 Using only the 3010 genes with 1-to-1 orthologs across all species could impact our ability to
255 detect convergent changes since we only use a relatively small fraction of the total number of
256 transcripts in each assembly (23435 to 37847; Supplemental Table 8). To investigate more
257 genes, we mapped reads from all samples to genes from each species which had a reciprocal-
258 best-blast-hit between species-pairs (which includes the 1-to-1 orthologs analysed above). This
259 approach generated 10 different datasets (one for each species assembly), with between 15500
260 and 17583 genes. After filtering out genes with low expression (using cpm, see Methods) in
261 each dataset, this approach allowed us to examine between 2.43-3.12 (dependent on species
262 and tissue) times more genes than using the 1-to-1 orthologs (Supplemental Table 8). Results
263 from this approach qualitatively confirmed the results found using only the 1-to-1 orthologs: the
264 percentage of genes showing a convergent expression ranged from 4-5% for whole-body
265 samples and 6-8% for the reproductive tract and leg samples, dependent on which of the

species transcriptome was used (Supplemental Table 8), and GSEA produced similar enriched GO terms (Supplemental Tables 9-11)

Species-pair specific changes

The approach taken above allowed us to identify genes which showed convergent changes in expression across independent transitions to asexuality. This approach will not identify expression changes confined to a single or few species-pairs. Such changes are clearly not convergent at the gene expression level, however, these changes could be convergent at the functional process level, whereby species-pair specific changes in gene expression are involved in common functional processes between species-pairs. To test this, we compared each asexual species to its closest sexual relative and called differentially expressed (DE) genes from each pairwise comparison.

The number of significantly DE genes between each pair varied greatly depending on species-pair and tissue (59 to 626, Supplementary Fig. 5), with a generally greater number of genes DE in leg tissue likely due to the smaller variation between replicates (common biological coefficient of variation was lowest for legs: whole-body = 0.314, reproductive tract = 0.340, legs = 0.238). There were no genes that showed overlap between all sexual-asexual species-pairs in any tissue (Fig. 5A). Examination of overlaps between pairs of sexual-asexual species-pairs found some overlapping genes, but these were close to the expectation by chance (Fig. 5B, for all levels see Supplemental Table 12). The majority of the DE genes also showed a significant interaction between species-pair and reproductive mode in the model used to identify convergently changing genes (whole-body = 69%, reproductive tract 66%, and legs = 81%), corroborating the finding that the vast majority of the DE genes are species-pair specific. Note the species-pair by reproductive mode interactions do not appear to be generated by one specific species-pair as generally genes DE between one species-pair were not DE between the other 4 species-pairs.

The DE genes of the different species pairs are not involved in convergent functional processes. Species-pair-specific genes were enriched for a number of GO terms, however no GO terms were found to overlap between all pairs in any tissue (Supplemental Fig. 6). Examination of overlaps between pairs of sexual-asexual species-pairs found some overlapping GO terms, but these were close to the expectation by chance (Supplemental Table 13). This pattern remained

300 even when a more liberal approach, whereby related GO-terms were considered as a unit, was
301 applied (Supplemental Fig. 7A). This overall lack of overlap suggests that species-pair-specific
302 genes are not involved in producing convergent phenotypes, but are instead the product of
303 either lineage-specific selection or drift. These two processes are difficult to disentangle, but our
304 results are more consistent with drift rather than lineage-specific selection. Indeed, species-pair
305 specific genes showed similar changes in gene expression across tissues, in contrast to the
306 mainly tissue-specific changes uncovered for convergently changing genes. The overlap of
307 species-pair specific genes between tissues was significantly greater than expected by chance
308 (Table 1).
309
310 Finally, these results were reproduced when examining a much larger set of genes (genes with
311 reciprocal-best-blast-hits between species-pairs, see above) as both genes DE between each
312 species-pair, and their enriched GO terms, showed little overlap (Supplemental Figs 8 and 9,
313 and Supplemental Tables 14 and 15).
314

Discussion

Asexuality has convergently evolved numerous times across the tree of life, and a large body of research focuses on the reasons why sexual reproduction persists in the face of competition from asexual lineages. By contrast, the molecular underpinnings of transitions from sexual to asexual reproduction remain largely unknown (Neiman et al. 2014). In this study we examined gene expression changes associated with transitions to asexuality across five independently evolved asexual lineages, in whole-bodies, reproductive tracts and legs. The changes we observe provide, for the first time, insights into the convergent evolution of asexuality at the molecular level.

We found evidence for convergent changes in gene expression in all three tissues. Three lines of evidence suggest that these changes are a product of selection. Firstly, parallel changes across multiple independent transitions represent strong evidence of selection and thus are unlikely to be due to drift (Losos 2011; Natarajan et al. 2016). Secondly, convergent changes were primarily tissue-specific. This finding is consistent with selection, because expression changes due to drift are likely to be correlated across tissues (Blekhman et al. 2008; Liang et al. 2018). Indeed, the different functional roles of reproductive tracts and legs make it unlikely that selection would drive changes in the same genes in all tissues. Finally, the functional processes of convergently expressed genes mirror the changes observed at the phenotypic level, supporting the interpretation that these genes contribute to the convergent phenotypic changes observed between sexual and asexual females.

The overall amount of convergence is striking, particularly in the reproductive tract and legs with approximately 8% of genes showing a convergent shift in expression. Such a large amount of convergence suggests that the path from sexual reproduction to asexuality is strongly constrained, requiring changes to the same genes and biological processes in order to produce asexual phenotypes.

Convergent changes in gene expression reveal the mechanisms underlying the production of asexual offspring

Asexuality is a complex adaptation that includes two major components: the ability to produce viable asexual offspring, and secondary adaptive changes that would not have been selected

349 for in sexual species (e.g. the reduction of costly sexual traits). A key change necessary for the
350 production of asexual offspring is the ability to produce unreduced eggs (Engelstädter 2008).
351 Convergently expressed genes in the reproductive tract were enriched for changes in meiosis,
352 and in particular meiotic spindles, which are key for the proper division of cells during meiosis.
353 Mutations in meiotic spindles have been shown to result in unreduced meiotic products in *D.*
354 *melanogaster*, and specifically in two genes (*bond* (Szafer-Glusman et al. 2008) and *pelo*
355 (Eberhart and Wasserman 1995)) which show convergent changes in expression in asexual
356 *Timema.* As such we suggest that these changes may underlie the non-reduction of eggs in
357 asexual *Timema.* An alternative hypothesis is that since *Timema* reproduce parthenogenetically
358 (and thus likely no longer recombine) changes in meiotic genes represent trait decay. Although
359 possible, previous work has shown that, in fact, meiotic genes are not only retained in asexual
360 lineages without damaging mutations, but often appear to be subject to selection for changes in
361 expression, via duplication or differential upregulation of promoters (Srinivasan et al. 2010;
362 Hanson et al. 2013; Raborn et al. 2016; Warren et al. 2018). Taken together with our results, we
363 suggest that modifications to meiotic genes, specifically those that that disrupt meiotic cell
364 division, are key in overcoming a major barrier to the evolution of asexuality: the production of
365 unreduced eggs.
366
367 The production of unreduced eggs is not the only barrier to producing offspring asexually. In
368 most species, sperm transfer essential components for the formation of a functioning
369 centrosome (Schatten 1994; Manandhar et al. 2005). This paternal contribution represents a
370 second key barrier in the evolution of parthenogenesis in many systems (Engelstädter 2008).
371 However, in phasmids the centrosome is assembled without any contribution from sperm in both
372 sexual and asexual species (Marescalchi et al. 2002). This may act as pre-adaptation for
373 asexuality in stick insects and account, in part, for the large number of asexual stick insects.
374
375 A final barrier to asexual offspring production in many systems is egg activation. In many
376 species mature oocytes are arrested at a specific stage (e.g. at metaphase II in mammals, and
377 metaphase I in most insects), and must be activated by sperm to re-enter the cell-cycle (Stricker
378 1999; Engelstädter 2008; Nishiyama et al. 2010). In insects however, egg activation does not
379 require sperm as activation is induced by the transit through the reproductive tract (Sartain and
380 Wolfner 2013). Despite this, ovulation and egg-laying rates are strongly tied to mating (Eberhard
381 1996; Gillott 2003) meaning this signal must be modified in order for asexual insects to have
382 normal levels of fecundity. In insects, the signal to a female that she has successfully mated is

383 likely detected by sensory neurons in her reproductive tract (Yapici et al. 2008). Consistent with
384 this, we find changes in gene expression linked to sensory neurons in the reproductive tract of
385 asexual females, which may act to cue high levels of ovulation without mating. Alternatively,
386 these changes may represent the decay of these neurons since they are no longer needed to
387 detect mating events, or these changes may result from cessation of sexual conflict. Sensory
388 neurons in the reproductive tract are known targets of substances in the male ejaculate (Heifetz
389 and Wolfner 2004; Sakai et al. 2009; Heifetz et al. 2014) to induce the release of eggs and to
390 reduce female receptivity (Gillott 2003). This manipulation is countered by female resistance
391 adaptations which are likely costly, meaning that, following a transition to asexuality, there will
392 be selection against them.
393
394 ***Convergent changes in gene expression show evidence for the decay of female sexual***
395 ***traits***
396
397 Sexual traits in asexual females are often observed to be reduced or lost (van der Kooi and
398 Schwander 2014). For instance, in insects, females typically produce pheromones as a sexual
399 cue to attract males (Greenfield 2002), and this cue has been repeatedly reduced or lost in
400 several asexual species (see van der Kooi and Schwander 2014), including *Timema*
401 (Schwander, Crespi, et al. 2013). Such trait decay can be the result of either reduced purifying
402 selection acting on traits that are now selectively neutral, or selection to reduce the cost of
403 producing sexual traits. In asexual *Timema* reproductive decay has been primarily attributed to
404 selection rather than reduced purifying selection, as reproductive trait decay in very young
405 asexual lineages is as extensive as in old ones (Schwander, Crespi, et al. 2013).
406
407 Convergent gene expression changes underlying the decay of reproductive traits are mostly
408 observed in *Timema* whole-bodies. In particular, we find enrichment of terms associated with
409 sperm storage and sexual behaviour. Changes in the legs were less obviously associated with
410 reproductive trait decay, however we do find changes in genes involved in cuticle development,
411 pigment biosynthesis, sensory perception of touch, and changes in sexual behavior. These
412 changes could represent the reproductive decay of both sexual cues (e.g. cuticular
413 hydrocarbons and pigmentation which are both important for mate choice in insects (reviewed in
414 Hunt and Sakaluk 2014), and their detection (via sensory receptors on the leg (reviewed in
415 Dahanukar et al. 2005)). In addition, we also find changes in genes associated with sex
416 determination in the soma, including *sex-lethal*, a master-feminizing switch in *Drosophila* (Cline

et al. 2010) which may have a major influence the development of many sexual traits in the legs.

Although we focus on expression, it is possible that the decay of sexual traits is also evident at the sequence level. By examining the coding regions of genes, we found evidence for reduced purifying selection acting on the sequence of genes showing convergent expression changes in the whole-body. This suggests, that in some cases, the reduction of sexual traits may be accomplished by both expression and sequence changes, which potentially act interactively to produce a phenotypic change.

Unexpectedly, we also find changes to immune function in the legs and whole-body, the majority of which show down-regulation in asexual females. A possible explanation for this is that asexual females are likely to face a reduced number of immune challenges compared to sexual females due to the elimination of sexually transmitted diseases, the costs of which can be considerable, even shaping the evolution of many aspects of an organism's life history, such as mate choice, mating rate, and sexual signal investment (Kokko et al. 2002; Knell and Webberley 2004). As such we suggest asexual females may be reducing the allocation of resources to immune function due to the absence sexually transmitted diseases. This effect may be particularly strong in solitary species such as *Timema*, where the majority of socially transmitted diseases come from sexual interactions.

Species-pair specific changes

In addition to convergent changes, we also identified many species-pair specific gene expression changes. In contrast to convergent genes, species-pair specific genes showed common shifts in expression across tissues, and inconsistent associations with functional processes between species-pairs, that were largely unrelated to asexual phenotypes. Taken together, these results suggest that the majority of changes we observe from a single sex-asex species-pair comparison are due to drift rather than selection. Our findings thus highlight the problem of drawing inferences on the causes or consequences of asexuality from the examination of only a single transition to asexuality, whereas examining several transitions allows us to disentangle adaptive changes and those due to drift.

Overall, we find evidence for a striking number of convergent changes across five transitions to asexuality. The amount of molecular convergence to expect, however, is dependent on several factors including the complexity of the phenotype, and the size of the mutational target (Martin and Orgogozo 2013). For instance, here we find that a key change required for asexual reproduction, the production of unreduced eggs, likely requires changes to meiotic spindle regulation. The pathways that govern meiotic spindle regulation are relatively small in number (Bennabi et al. 2016), meaning that only a small minority of genes are likely able to confer the relevant changes, making the chance of convergence for this trait relatively high. In contrast, the observed reduction of sexual traits could be produced by changes to numerous genes and pathways (i.e. there is a large mutational target) making convergent changes for these traits less likely. Despite this, our and previous studies examining trait loss have also demonstrated a high amount of convergence (Whittall et al. 2006; Gross et al. 2009; Partha et al. 2017; Sackton et al. 2018), implying that certain genes have a disproportionate role in not only the convergent evolution of novel phenotypes, but also in their convergent loss (Martin and Orgogozo 2013; Stern 2013).

476 # Materials and Methods

477

478 ***Samples***

479

480 Females for whole-body samples were collected from the field as juveniles in spring 2013. All
481 individuals were then raised in common garden conditions (23°C, 12h:12h, 60% humidity, fed
482 with Ceanothus cuttings) until eight days following their final molt. Prior to RNA extraction,
483 individuals were fed with artificial medium for two days to avoid RNA contamination with gut
484 content and then frozen at -80°C. Individuals used for tissue-specific samples were collected in
485 spring 2014 as juveniles and raised in the same common-garden conditions as whole-body
486 samples. For leg samples three legs were used from each individual (one foreleg, one midleg,
487 and one hindleg). Reproductive tracts were dissected to consist of ovaries, oviducts and
488 spermatheca. Note the same individuals were used for leg and reproductive tract samples.
489 Collection locations for all samples are given in Supplemental Table 16.

490

491 ***RNA extraction and sequencing***

492

493 The three biological replicates per species and tissue consisted of 1-9 individuals per replicate,
494 which were combined prior to RNA extraction (207 individuals in 90 replicates in total; see
495 Supplemental Table 16). RNA extraction was performed by freezing individuals in liquid nitrogen
496 followed by addition of Trizol (Life Technologies) before being homogenized using mechanical
497 beads (Sigmund Lindner). Chloroform and ethanol were then added to the samples and the
498 aqueous layer transferred to RNeasy MinElute Columns (Qiagen). RNA extraction was then
499 completed using an RNeasy Mini Kit following the manufacturer's instructions. RNA quantity and
500 quality was measured using NanoDrop (Thermo Scientific) and Bioanalyzer (Agilent). Strand-
501 specific library preparation and single-end sequencing (100 bp, HiSeq2000) were performed at
502 the Lausanne Genomic Technologies Facility.

503

504 The 90 libraries produced a total of just over 3 billion single-end reads. Four whole-body and six
505 tissue-specific libraries produced significantly more reads than the average for the other
506 samples. To reduce any influence of this on downstream analyses, these libraries were sampled
507 down to approximately the average number of reads for whole-body or tissue-specific libraries
508 respectively using seqtk (https://github.com/lh3/seqtk Version: 1.2-r94).

509

510 ***Transcriptome references***

511

512 *De novo* reference transcriptome assemblies for each species were generated previously (Bast
513 et al. 2018). For our analyses we used the 3010 one-to-one orthologs present in all 10 *Timema*
514 species as identified by Bast et al. (2018). Identified ortholog sequences varied in length among
515 different species. Since length variation might influence estimates of gene expression, we
516 aligned orthologous sequences using PRANK (v.100802, default options) (Löytynoja and
517 Goldman 2005) and trimmed them using alignment_trimmer.py (Parker 2016) to remove
518 overhanging gaps at the ends of the alignments. If the alignment contained a gap of greater
519 than 3 bases then sequence preceding or following the alignment gap (whichever was shortest)
520 was discarded. Three genes were discarded at this stage as the trimmed length of sequence
521 was <300 bp. These trimmed sequences were then used as reference transcriptomes for read
522 mapping. Note that genes with significant Blast hits to rRNA sequences were removed prior to
523 mapping.

524

525 ***Read trimming and mapping***

526

527 Raw reads were trimmed before mapping. Firstly CutAdapt (Martin 2011) was used to trim
528 adapter sequences from the reads. Reads were then quality trimmed using Trimmomatic v 0.36
529 (Bolger et al. 2014): first clipping leading or trailing bases with a phred score of <10 from the
530 read, before using a sliding window from the 5' end to clip the read if 4 consecutive bases had
531 an average phred score of <20. Following quality trimming any reads <80 bp in length were
532 discarded. Surviving reads from each library were then mapped separately to the reference
533 transcriptome using Kallisto (v. 0.43.1) (Bray et al. 2016) with the following options -l 210 -s 25 -
534 -bias --rf-stranded for whole-body samples and -l 370 -s 25 --bias --rf-stranded for tissue specific
535 samples (the -l option was different for whole-body and tissue specific samples as the fragment
536 length for these libraries was different).

537

538 ***Differential expression analysis***

539

540 Expression analyses were performed using the Bioconductor package EdgeR (v. 3.18.1)
541 (Robinson et al. 2010) in R (v. 3.4.1) (R Core Team 2017). Analyses were done separately for
542 each tissue. Genes with counts per million less than 0.5 in 2 or more libraries per species were
543 excluded from expression analyses. Normalization factors for each library were computed using

544 the TMM method in EdgeR. To estimate dispersion we then fit a generalized linear model (GLM)
545 with negative binomial distribution with the terms species-pair, reproductive mode and their
546 interaction. We used a GLM likelihood ratio test to determine significance of model terms for
547 each gene by comparing appropriate model contrasts. P-values were corrected for multiple tests
548 using Benjamini and Hochberg's algorithm (Benjamini and Hochberg 1995), with statistical
549 significance set to 5%. Using this approach, we classified genes as convergently differentially
550 expressed when there was a significant effect of reproductive mode (FDR < 0.05) but no
551 interaction effect of species-pair by reproductive mode (FDR > 0.05). Differentially expressed
552 genes within each species-pair were identified using pairwise contrasts between each sexual
553 and asexual pair.
554
555 To determine if genes DE within each species-pair and tissue show greater than expected
556 number of overlapping genes we used the SuperExactTest package (v. 0.99.4) (Wang et al.
557 2015) in R which calculates the probability of multi-set intersections. When examining multiple
558 intersections p-values were multiple test corrected using Benjamini and Hochberg's algorithm
559 implemented in R.
560
561 To test if the observed number of convergent genes was significantly greater than expected by
562 chance we performed a permutation test by whereby, for the read counts of each gene, we
563 randomly switched the assignment of reproductive mode (sexual or asexual) within a species-
564 pair. Note that all biological replicates from a particular group were always assigned to the same
565 reproductive mode (i.e. In the event of a switch, all sexual replicates were assigned as asexual,
566 and vice versa). This process was repeated to produce 10,000 permuted data sets, which were
567 then ran through the gene expression pipeline described above to generate a distribution of the
568 number of convergent genes we expect to find by chance.
569
570
571 ***Go term analysis***
572
573 Genes were functionally annotated using Blast2GO (version 4.1.9) (Götz et al. 2008) as follows:
574 sequences from each sexual species were compared with BlastX to either NCBI's nr-arthropod
575 or *Drosophila melanogaster* (drosoph) databases, keeping the top 20 hits with e-values <1 x 10^{-}
576 ^{3}. Interproscan (default settings within Blast2GO) was then run for each sequence, and the
577 results merged with the blast results to obtain GO terms. This produced two sets of functional

578 annotations, one derived from all arthropods and one specifically from *Drosophila melanogaster*.
579 The *D. melanogaster* GO term annotation generated around four times more annotations per
580 sequence than NCBI's nr-arthropod database. We therefore conducted all subsequent analyses
581 using the GO terms derived from *D. melanogaster*, but note that results using the annotations
582 from all arthropods were qualitatively the same (see Supplementary Fig. 7B).
583
584 We conducted gene set enrichment analyses using the R package TopGO (v. 2.28.0) (Alexa
585 and Rahnenfuhrer 2016) using the elim algorithm to account for the GO topology. Gene set
586 enrichment analyses identify enriched GO terms in a threshold-free way, by finding GO-terms
587 that are overrepresented at the top of a ranked list of genes. For comparisons within a species-
588 pair, genes were ranked by FDR; to identify enrichment of convergent genes, genes were
589 ranked by FDR value for reproductive mode, with the FDR value for genes that showed a
590 significant lineage by reproductive mode set to 1. GO terms were considered to be significantly
591 enriched when $p < 0.05$. Enriched GO terms were then semantically clustered using ReviGO
592 (Supek et al. 2011) to aid interpretation.
593
594 The significance of overlapping GO terms was determined using SuperExactTest as described
595 above. The hierarchical nature of GO terms generates a bias towards finding a significant
596 amount of overlap, since enrichment terms are non-independent. It is however possible that the
597 complexity of the GO term hierarchy could lead to convergent functional processes being
598 overlooked. For instance if a GO term is enriched in one comparison, but its parent term is
599 enriched in another comparison, then there would be no apparent overlap. To address this, we
600 also looked at the amount of 'linked overlap' of GO terms, whereby significant GO terms were
601 first clustered together based on parent or child terms.
602
603 For the GO term enrichment analyses of convergently differential expressed genes we used
604 only the annotation from *T. bartmani* as it had the most number of sequences annotated.
605 Annotations to each of the other species were very similar to those from *T. bartmani,* with 80%
606 of annotations being identical across all 5 species annotations. The remaining 20% of
607 sequences were typically characterized by an additional term in one or more of the species. For
608 comparisons within a lineage we used the annotation of the sexual species in that lineage.
609 Although the annotations are very similar across all ten species the small differences in
610 annotation could create differences in the amount of overlap observed between contrasts (e.g. if
611 a term is annotated to an ortholog in one annotation but not another). To examine this, we

612 repeated the analysis using only annotations from *T. bartmani*. This produced a virtually
613 identical result (Supplemental Fig. 7C) as when using the species-pair specific annotations.
614
615 **Polymorphism and divergence**
616
617 To test for differences in the rate of evolutionary divergence between gene categories, we used
618 dN/dS ratios for each of the one-to-one orthologs from (Bast et al. 2018). To obtain an estimate
619 for pN/pS reads from the whole-body libraries for each asexual species were mapped to the
620 reference using RSEM/bowtie2 with default parameters and fragment length mean = 200
621 fragment length sd = 100 (Li and Dewey 2011; Langmead and Salzberg 2012). Samtools v1.2
622 was then used to create an mpileup file, which was filtered with VarScan v2.3.2 (minimum
623 coverage = 20, minor allele frequency = 10%, and minimum average phred quality = 20) to
624 obtain SNPs. To identify nonsynonymous and synonymous segregating polymorphisms we
625 identified the n-fold degenerate positions following Li et al. (1985) from which pN, pS and
626 (pN/pS) could be calculated per gene. Comparison of mean pN/pS and dN/dS between
627 convergent and non-convergent (background) genes was conducted using a permutation t-test
628 (number of permutations = 10000) in R.
629
630 ***Cross-species mapping***
631
632 All of the above analyses used only the one-to-one orthologs. To examine a larger fraction of
633 the transcriptome we produced species-pair references by using a reciprocal blast between the
634 assemblies of sexual-asexual sister species (blastN, minimum e-val = 0.00001, minimum query
635 coverage = 30%). Prior to this step potential contaminants were filtered from these by blasting
636 transcripts to local versions of the nt (using blastN, default options except task blastn,
637 max_target_seqs = 10) and nr (using blastX, default options except, max_target_seqs = 10)
638 databases (downloaded: 07/08/2016) using NCBI's blast client (v. 2.2.30+). Blast hits with an e-
639 value > 0.0000001 were discarded. The remaining blast hits were used to assign a phylum to
640 sequences if >=50% of Blast hits came from one phylum (in the event of a tie, the taxa with the
641 highest e-value was used as a tiebreaker). Transcripts that were assigned to a non-arthropoda
642 phylum were discarded (note that transcripts with no Blast hits or that blasted to mixed phyla
643 were retained). This filtering removed between 4-8% of transcripts (see Supplemental Table 8).
644 Reads of each species were then mapped to each species-pair references in the same way as

645 for the 1-to-1 orthologs. Differential expression analyses and GO-term enrichment analyses
646 were then repeated as described above.
647
648
649
650

651 ## Data

652

653 Raw reads have been deposited in the SRA. Accession codes are given in Supplementary
654 Table 16. Scripts for the analyses in this paper are available at:
655 https://github.com/DarrenJParker/Timema_convergent_gene_expression.

656

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658

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662

663 ## Author contributions

664

665 T.S., D.J.P. and K.J. designed the study. Z.D., K.J. and TS collected samples, performed
666 dissections and molecular work. D.J.P. analysed the data with input from J.B., T.S. and M.R.R.
667 D.J.P. and T.S. wrote the manuscript with input from all authors.

668

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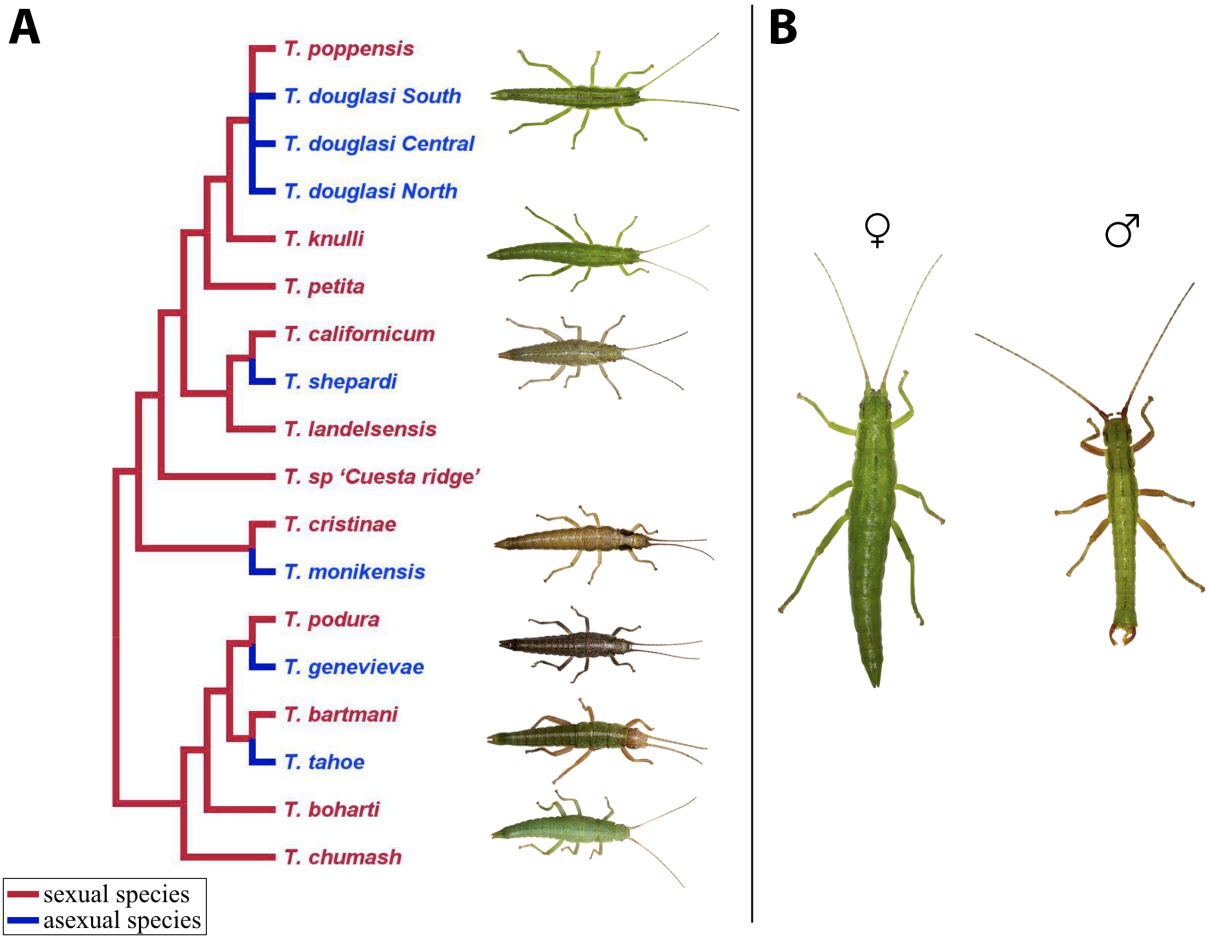
860

Table 1 | Overlap of differentially expressed genes between different tissue types. Number of genes expected by chance given in parentheses. P-values are from a fisher's exact test corrected for multiple tests. Species names are abbreviated as follows: Tbi = *T. bartmani*, Tce = *T. cristinae*, Tps = *T. poppensis*, Tcm = *T. californicum*, Tpa = *T. podura*, Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.

	Reproductive tract & legs	**Whole-body & legs**	**Whole-body & reproductive tract**
Convergent genes	22 (16) p = 0.14	7 (5) p = 0.21	4 (4) p = 0.65
Species-pair (Tbi-Tte)	62 (24) p = 1.30 $\times 10^{-14}$	68 (29) p = 4.57 $\times 10^{-14}$	25 (7) p = 4.53 $\times 10^{-9}$
Species-pair (Tce-Tms)	88 (47) p = 1.36 $\times 10^{-10}$	48 (9) p = 6.19 $\times 10^{-25}$	47 (14) p = 3.86 $\times 10^{-16}$
Species-pair (Tcm-Tsi)	42 (11) p = 4.45 $\times 10^{-15}$	24 (5) p = 4.64 $\times 10^{-11}$	19 (3) p = 4.64 $\times 10^{-11}$
Species-pair (Tpa-Tge)	153 (134) p = 1.99 $\times 10^{-2}$	102 (60) p = 1.22 $\times 10^{-9}$	86 (54) p = 8.75 $\times 10^{-07}$
Species-pair (Tps-Tdi)	56 (20) p = 1.31 $\times 10^{-15}$	59 (37) p = 6.08 $\times 10^{-5}$	32 (7) p = 7.81 $\times 10^{-14}$

871 **Figures**



872
873 **Figure 1** | **A.** Phylogeny of described *Timema* species (redrawn from (2017) with asexual
874 species added from (2011)). Sexually reproducing species are shown in red, independently
875 derived asexual lineages in blue. Our study used the five asexual species (for *T. douglasi* only
876 the southern lineage was used) and their sexual sister species. **B.** Sexual dimorphism in
877 Timema (*T. knulli*).
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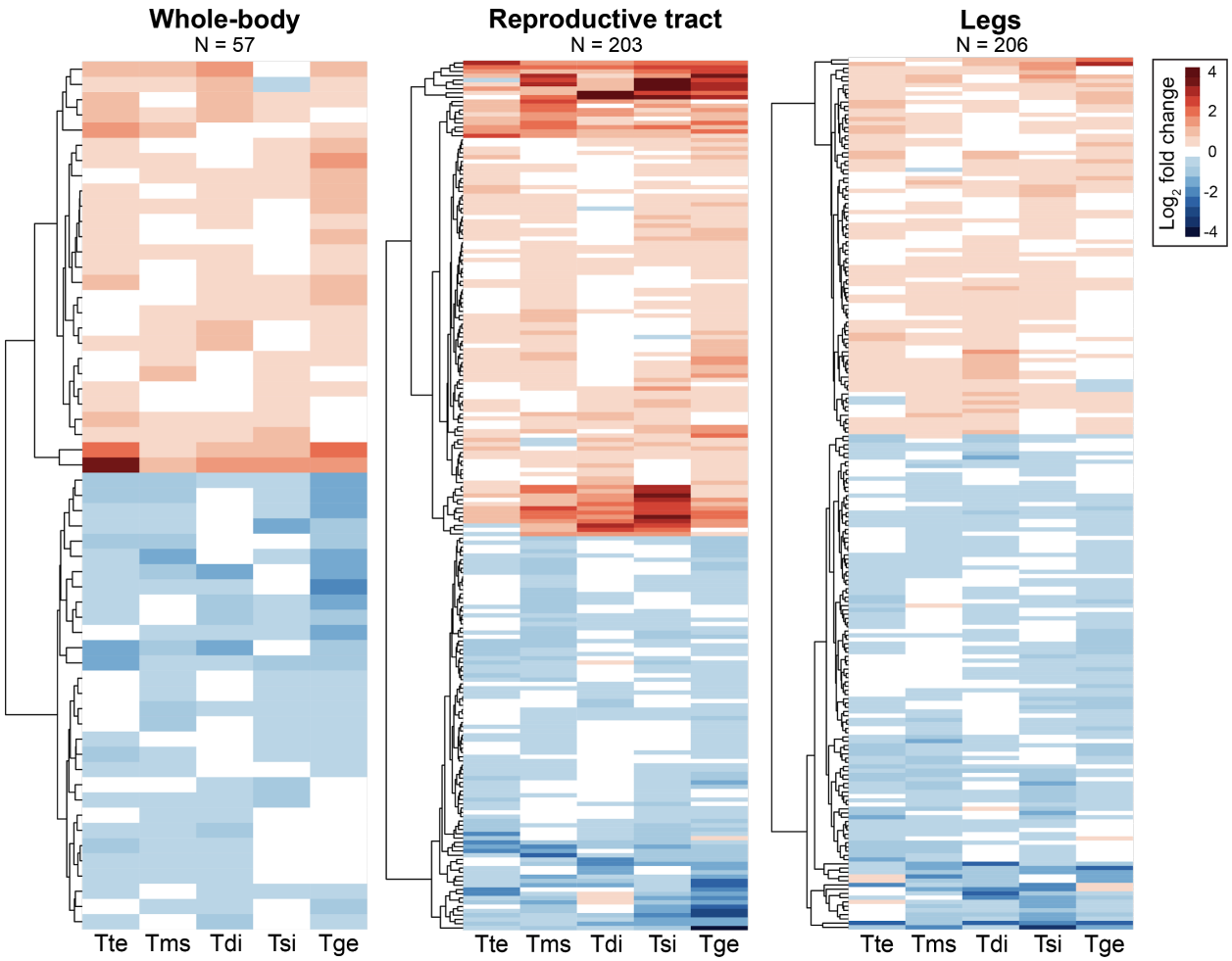


Figure 2 | Heatmaps of genes showing convergent gene expression changes between sexual and asexual females for whole-bodies, reproductive tract, and legs. Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.

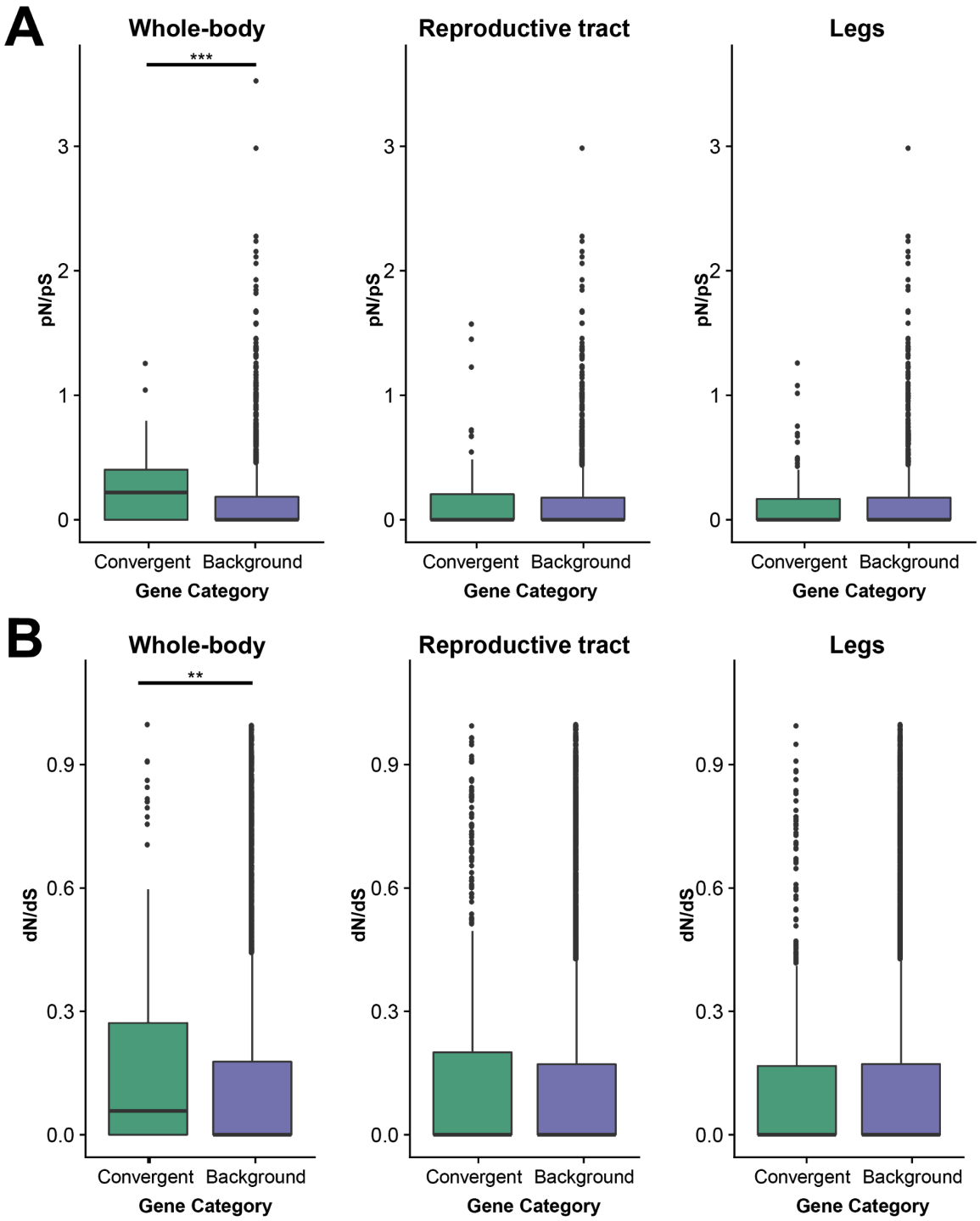


Figure 3 | pN/pS ratios (A) and dN/dS ratios (B) for convergently expressed genes versus all other genes expressed in that tissue for whole-bodies, reproductive tracts and legs. Significance is indicated by asterisks (** < 0.01, *** < 0.001).

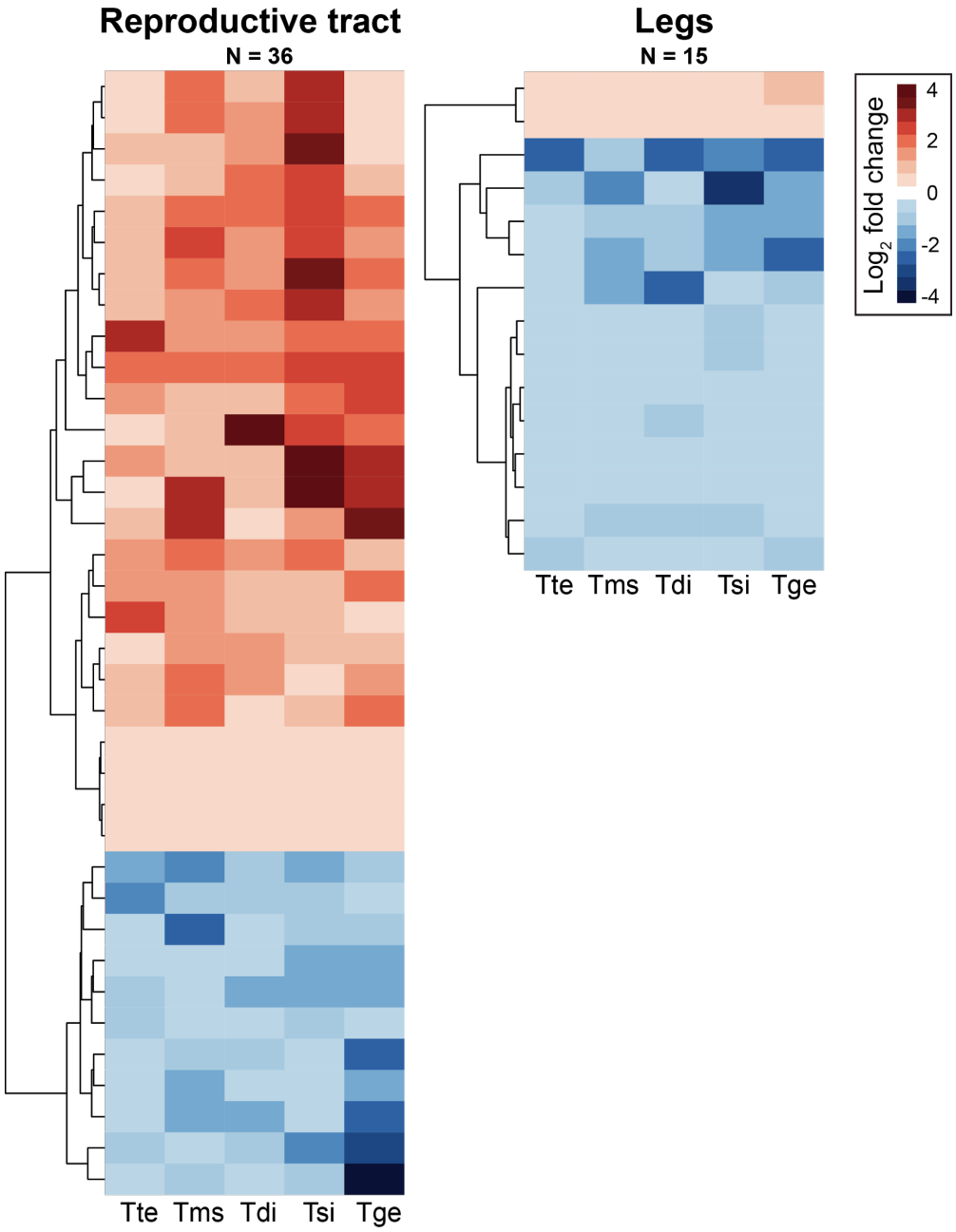


Figure 4 | Heatmaps of candidate convergent gene expression changes between sexual and asexual females for the reproductive tract and legs. Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.

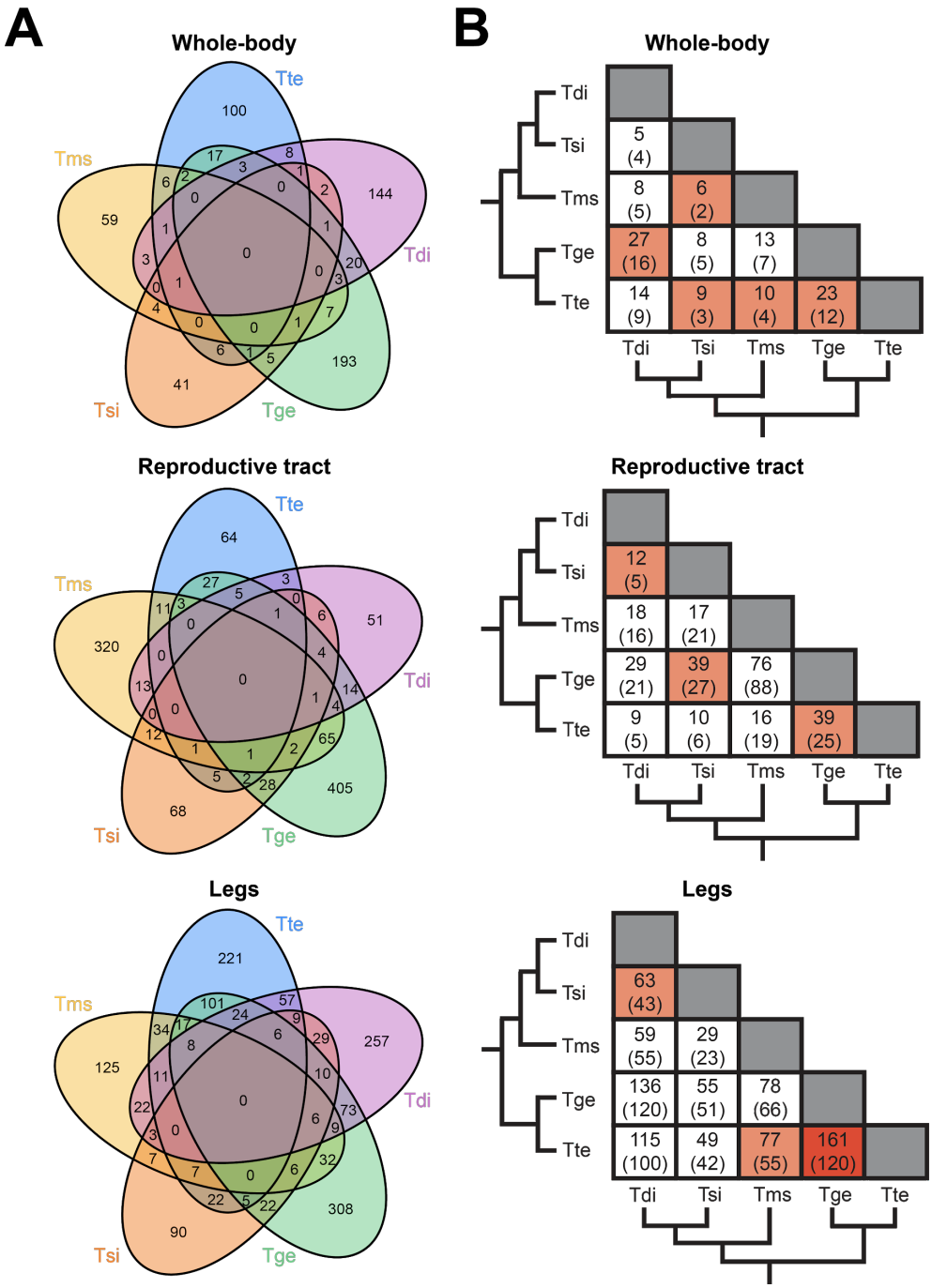
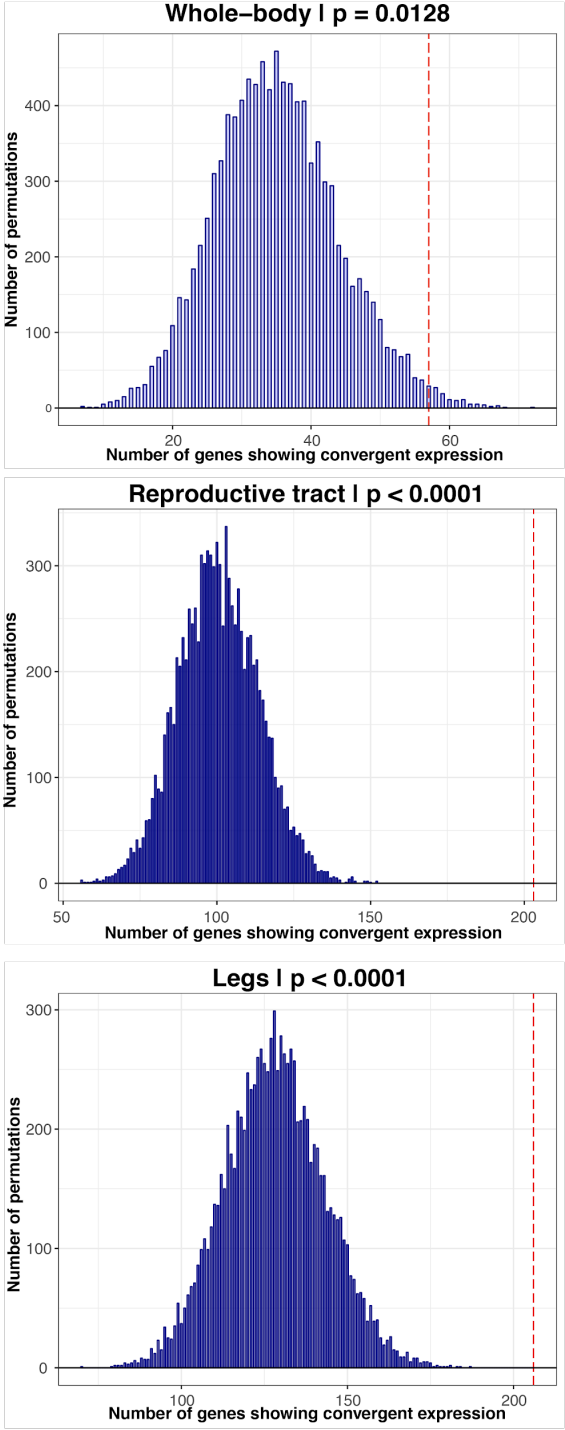
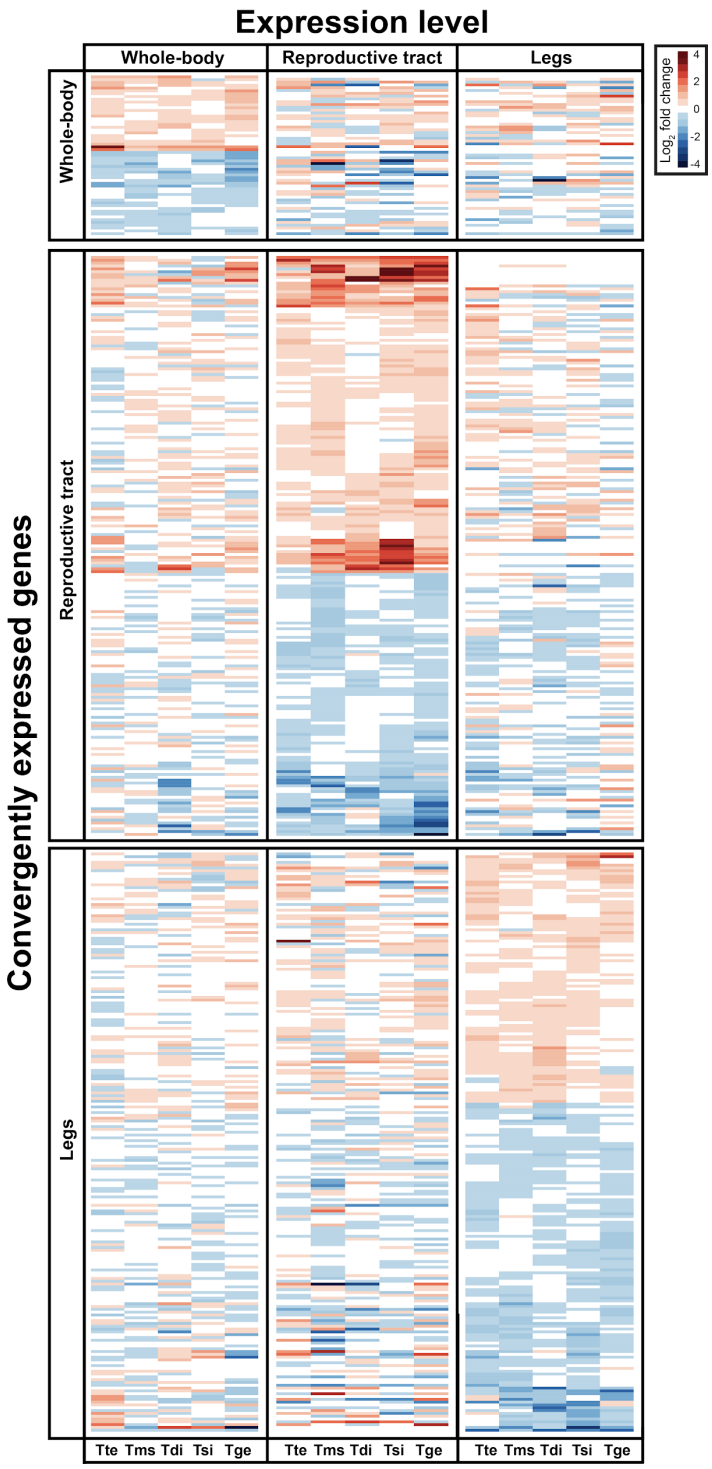


Figure 5 | A. Venn-diagrams showing the number differentially expressed (DE) genes between sexual and asexual females that are shared among species-pairs for whole-body, reproductive tract, and legs for 10 species orthologs (FDR < 0.05). B. Matrices showing pairwise overlap of DE genes between sex-asex species with the number of genes expected by chance given in parentheses. Colours represent a significantly greater overlap than expected by chance (red, FDR < 0.001, orange < 0.05). The phylogeny shows the relationships between asexual species (from (2011)). Species-pair names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.

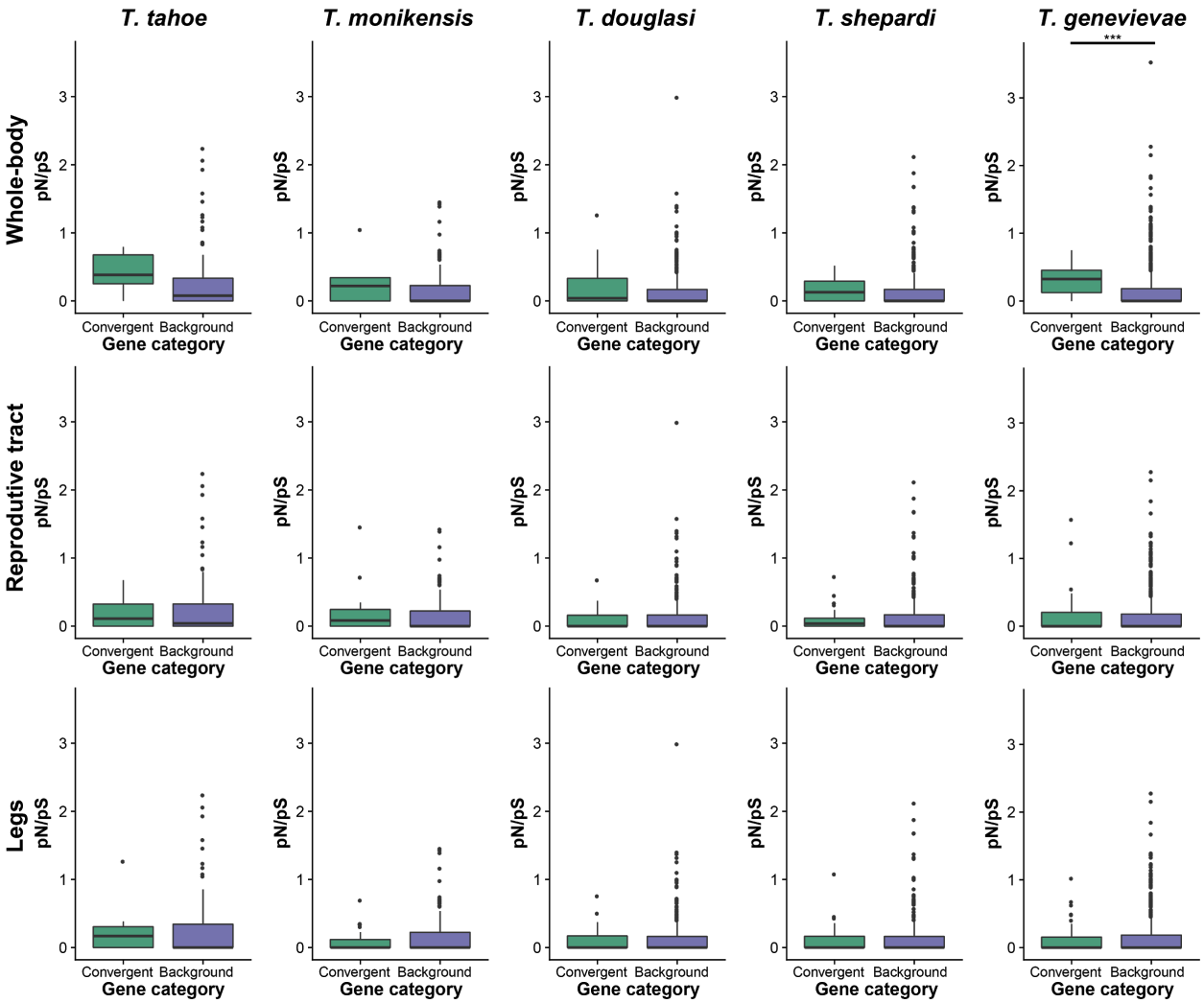
Supplementary Figures



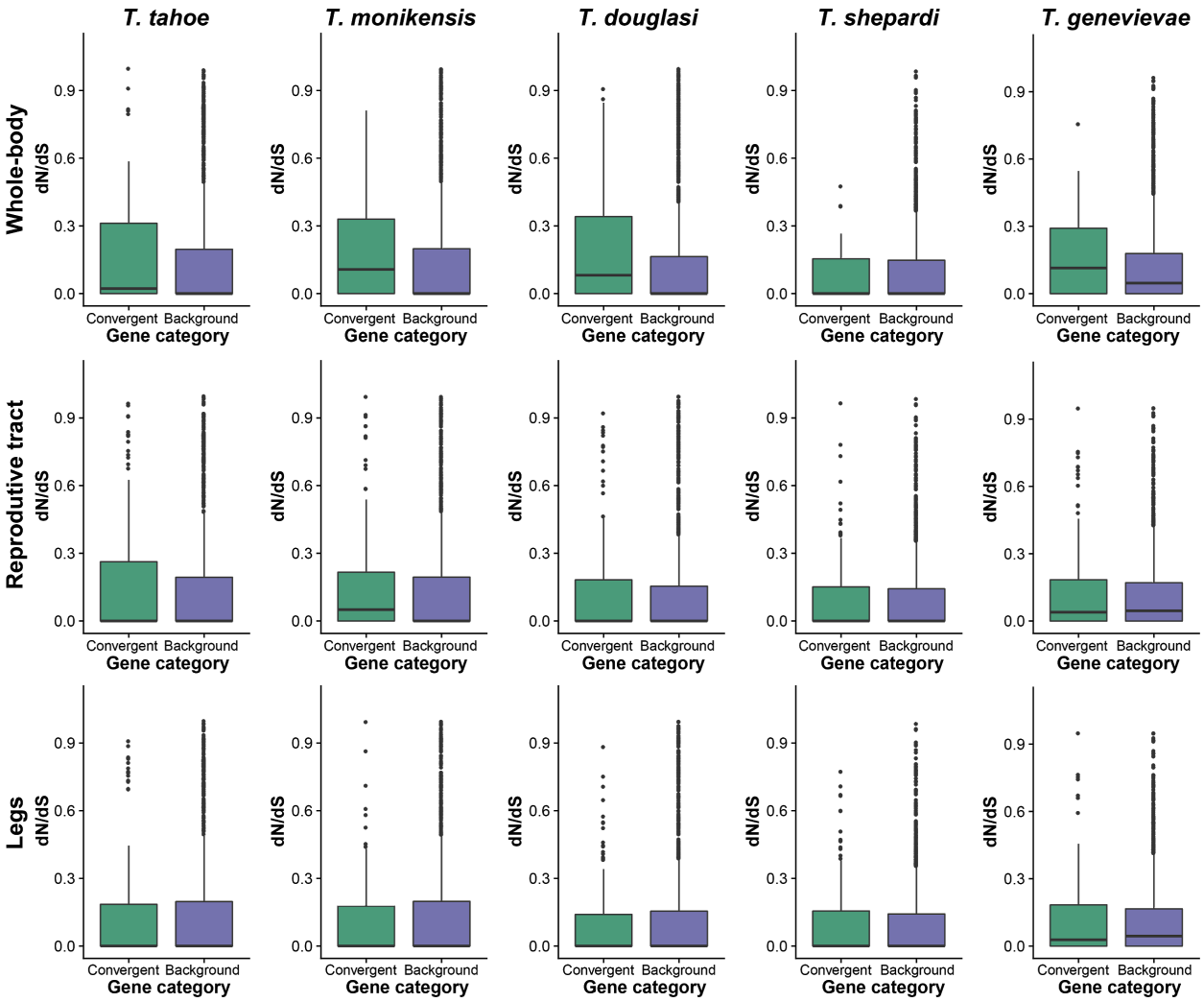
Supplementary Figure 1 | Number of genes expected to show a convergent expression pattern by chance (assessed by assigning reproductive mode randomly within species pairs for each gene for 10,000 permuted datasets). The observed number of convergent genes is indicated by the red dashed line. P-values refer to the probability of observing a number of convergent genes greater-than or equal-to the observed value.



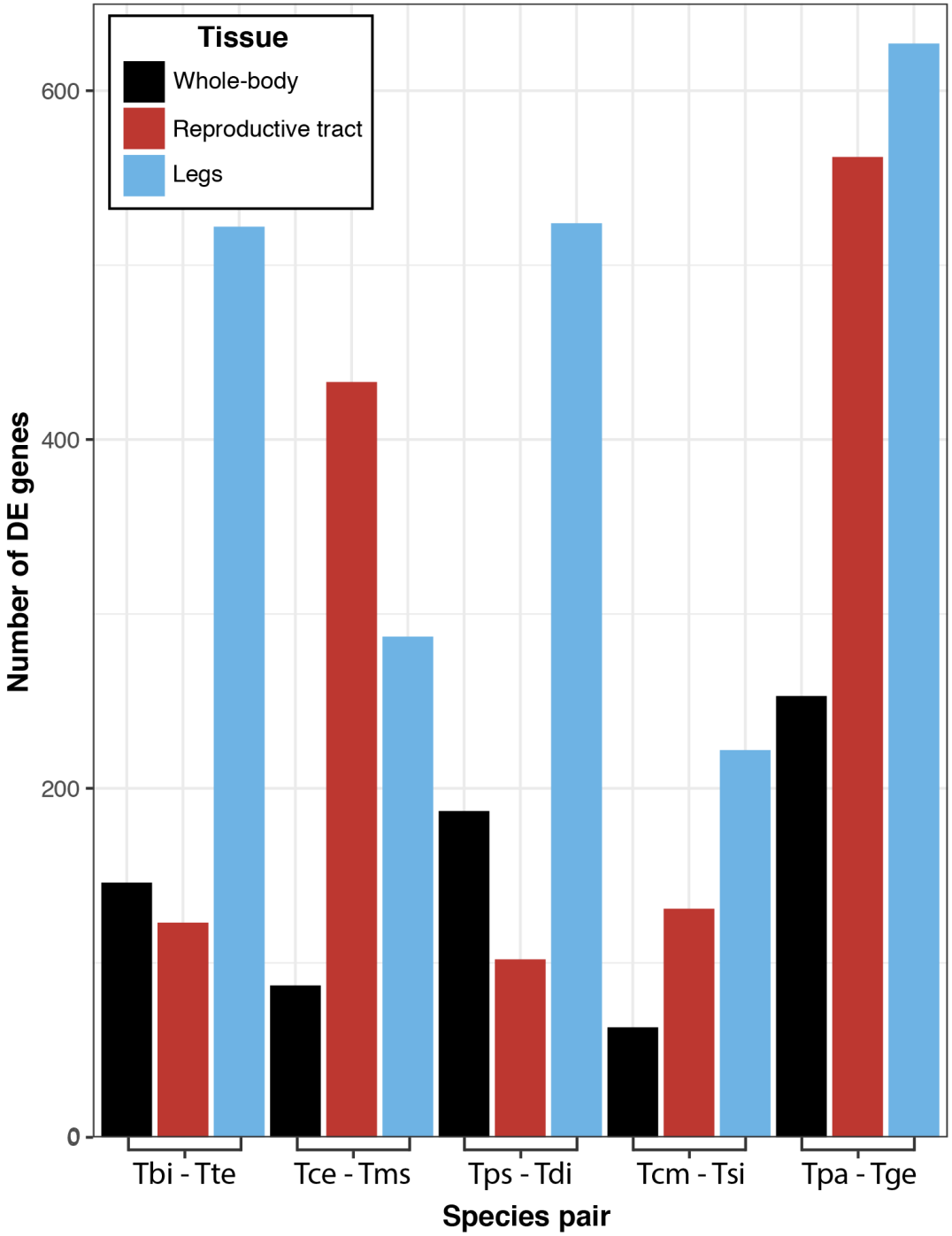
Supplementary Figure 2 | Convergently changing genes are largely tissue-specific. Heatmaps of genes showing convergent gene expression changes between sexual and asexual females for whole-bodies, reproductive tract, and legs, including their expression in other tissues. Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.



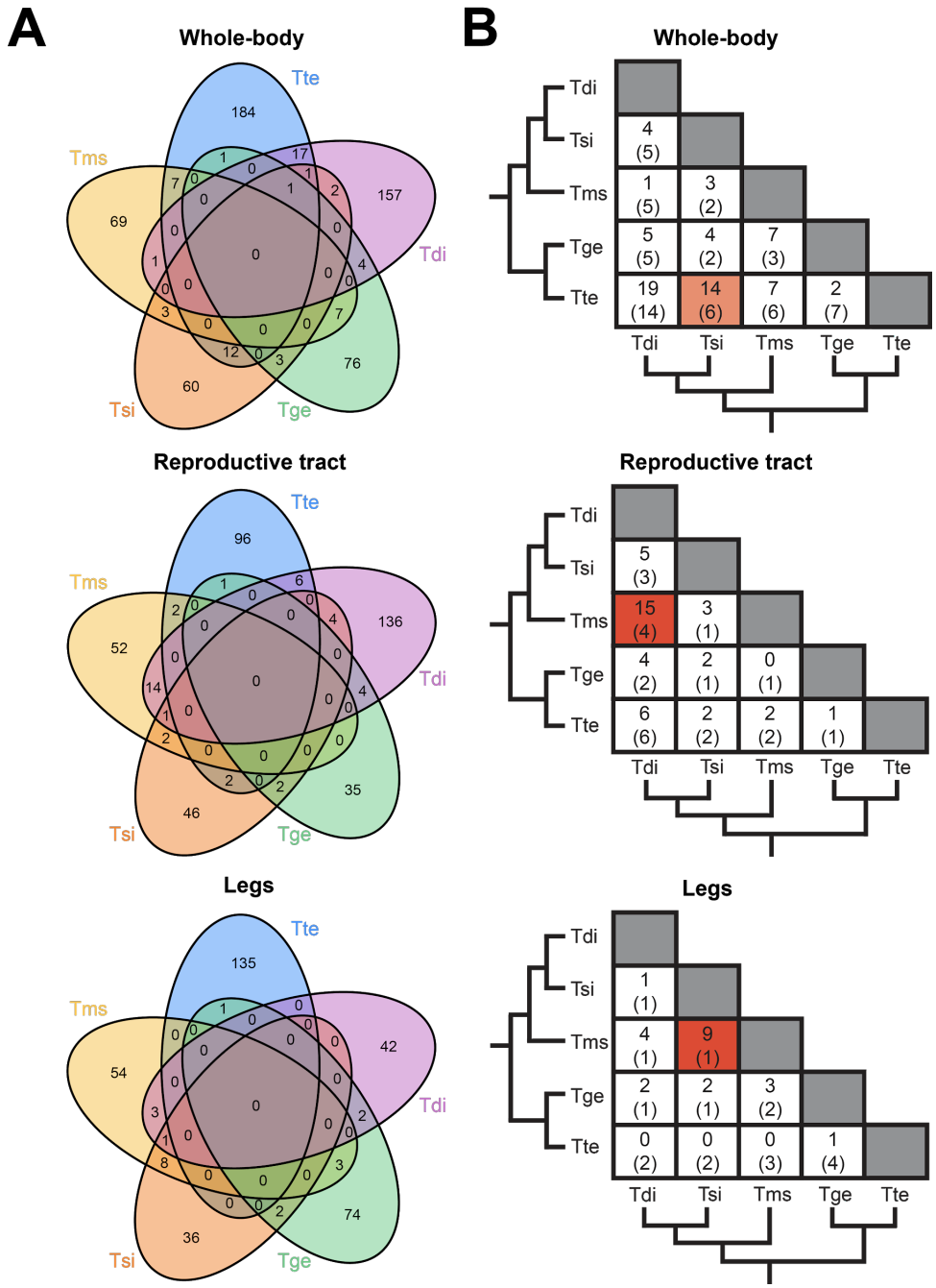
Supplementary Figure 3 | pN/pS ratios for convergently expressed genes versus all other genes expressed in that tissue for whole-bodies, reproductive tracts and legs for each asexual species. Significance is indicated by asterisks (*** < 0.001) from a Wilcoxon test.



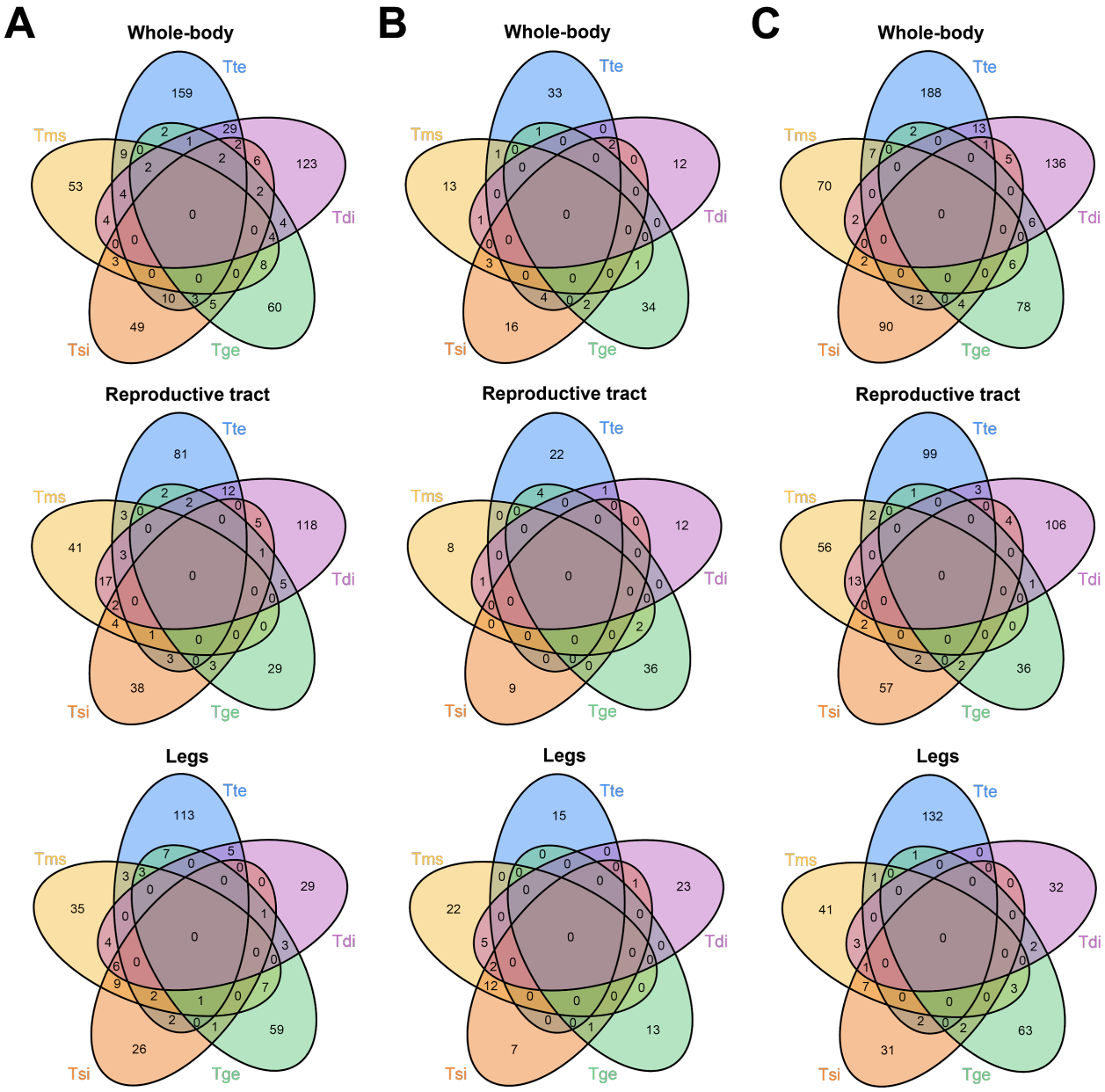
Supplementary Figure 4 | dN/dS ratios for convergently expressed genes versus all other genes expressed in that tissue for whole-bodies, reproductive tract and legs for each asexual species. No comparisons were significantly different (Wilcoxon test, $p > 0.05$).



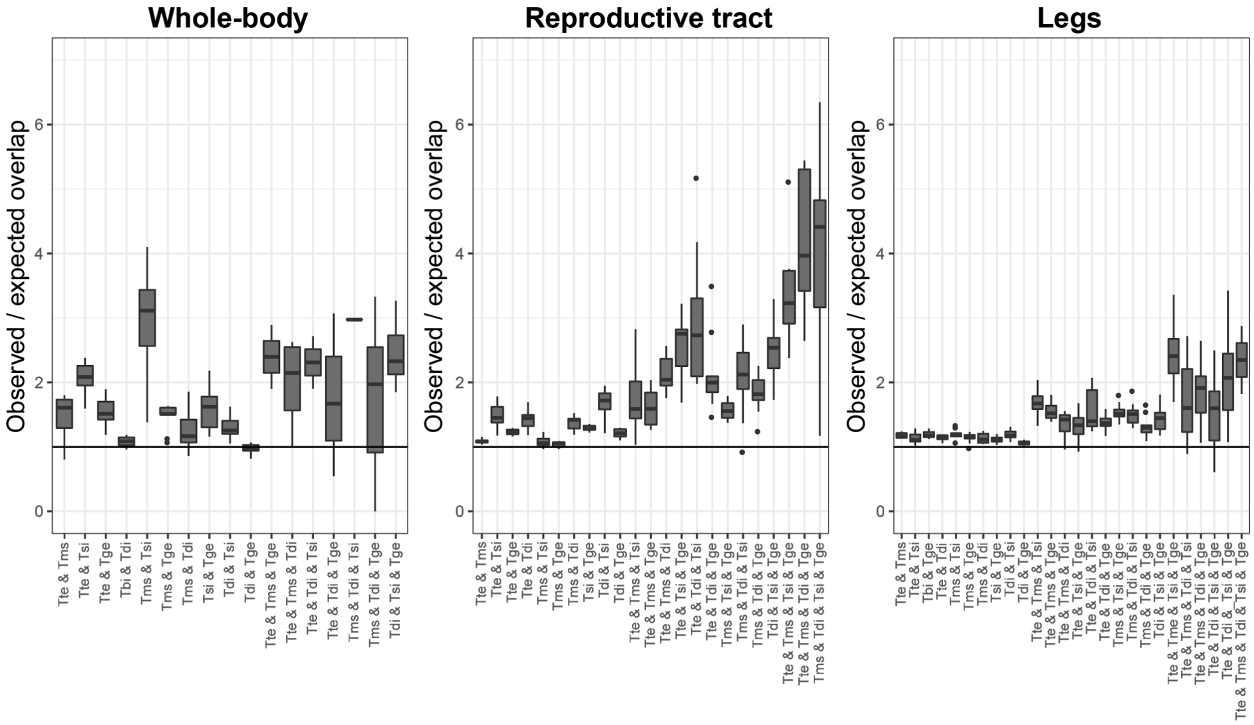
Supplementary Figure 5 | Number of DE genes (FDR < 0.05) between sexual and asexual females for each species-pair for whole-body, reproductive tract, and legs for the 10 species orthologs. Species names are abbreviated as follows: Tbi = *T. bartmani*, Tce = *T. cristinae*, Tps = *T. poppensis*, Tcm = *T. californicum*, Tpa = *T. podura*, Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.



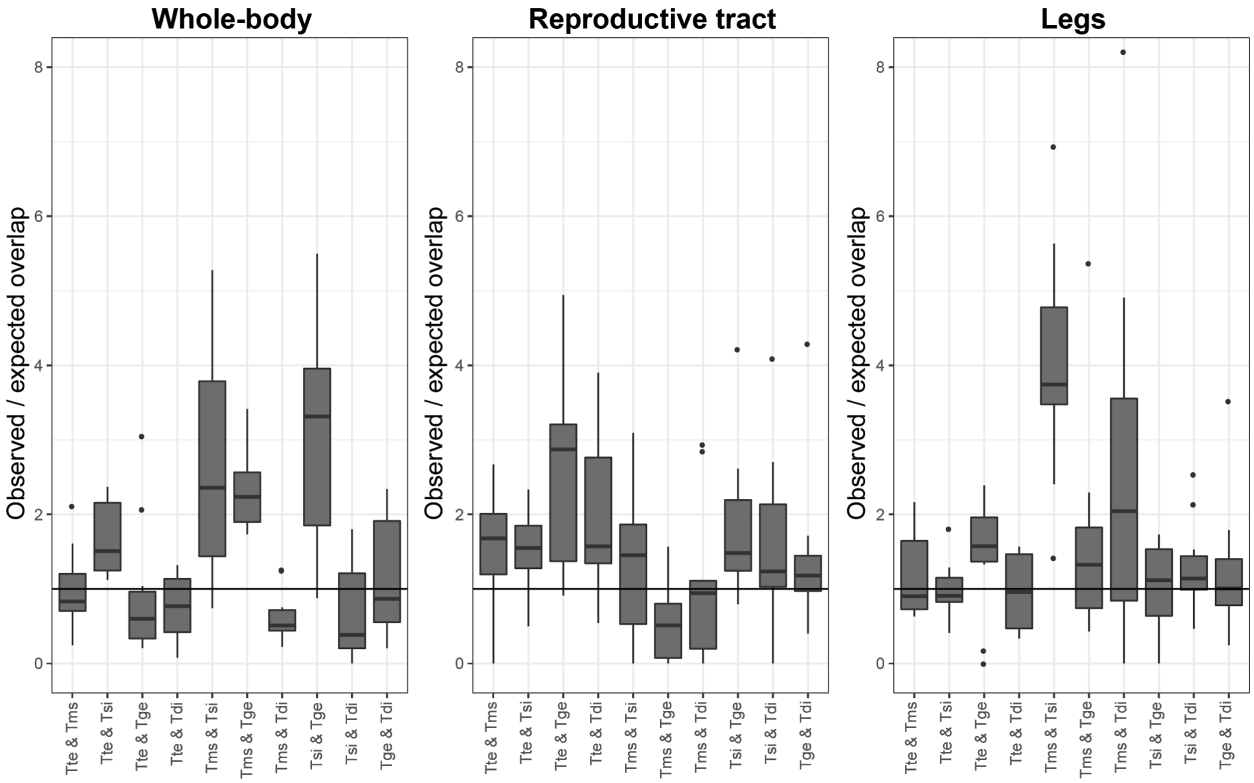
Supplementary Figure 6 | A. Venn-diagrams showing the number of enriched GO-terms between sexual and asexual females that are shared among species-pairs for whole-body, reproductive tracts, and legs for 10 species orthologs (FDR < 0.05). B. Matrices showing pairwise overlap of enriched GO-terms between sexual and asexual females with the number of GO terms expected by chance given in parentheses. Colours represent a significantly greater overlap than expected by chance (red, FDR < 0.001, orange < 0.05). The phylogeny shows the relationships between asexual species (from Schwander et al (2011)). Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.



Supplementary Figure 7 | Venn-diagrams showing the number of enriched GO-terms ($p < 0.05$) for differences between sexual and asexual females that are shared among species-pairs in the whole-body, reproductive tract, and legs when: **A** GO terms were first clustered together based on parent or child terms, **B** only NCBI's nr annotation was used, **C** only the *T. bartmani* annotation. Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.



Supplementary Figure 8 | Ratio of observed to expected amount of overlap for genes when reads were mapped to the whole transcriptome for each species. Note boxes are only shown when the expected overlap was >= 1. Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.



Supplementary Figure 9 | Ratio of observed to expected amount of overlap of enriched GO-terms when reads were mapped to the whole transcriptome for each species. Note boxes are only shown when the expected overlap was >= 1. Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*.